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A Simple and Fast Method for Derivation of blastocyst-like cell aggregates (BLCAs) from Murine Embryonic Stem Cells

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Abstract

The study of blastocyst differentiation from stem cells is still at its stage of infancy. In this study, a simple and efficient method was developed to derive blastocyst-like cell aggregates (BLCAs) from murine embryonic stem cells (mESCs). This was achieved by culturing the mESCs in non-adherent dishes. 0020BLCAs (13 ± 2 per million mESCs) were obtained in only 5 days and they were found to express the protein and gene markers of trophoblasts. Furthermore, the BLCAs showed the normal trophoblasts' capability of penetrating the uterine wall after being transferred into pseudo-pregnant mice, indicating the trophoblast-like functionality of the derived BLCAs. Collectively, we successfully developed a simple and fast 3D suspension culture approach to differentiate mESCs into BLCAs, which may provide important clues into the development of early embryos and stem cells. This may also offer a simple and efficient approach to isolate trophoblast cells for studying the native microenvironment of pluripotent stem cells without the need to sacrifice animals.

Keywords

Blastocyst; trophoblast; embryonic stem cells; pseudo-pregnant mice

1. INTRODUCTION

Stem cells have been widely studied in regenerative medicine, as they possess the pluripotent ability to differentiate into various cell types^{1,2}. The research on germ cell differentiation from stem cells is considered an attractive strategy to treat women suffering from infertility. More recently, research on derivation of eggs (e.g., oocytes and blastocysts)

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from the pluripotent embryonic stem cells has attracted increasing attentions an alternative method for accelerating animal breeding and production in the meat industry^{3,4}, as well as for potentially treating infertility⁵. However, research on this topic is still in the early stage; additionally, a long time (up to 45 days) is needed for pluripotent stem cells to differentiate into eggs^{6,7,8,9,10}

In this study, we developed a new and convenient method to differentiate murine embryonic stem cells (mESCs) into blastocyst-like cell aggregates (BLCAs) in only 5 days. The BLCAs were further confirmed by using protein and germ markers *in vitro*, and by transferring them into the uterine wall to examine their trophoblast-like functionality *in vivo*, for determining whether or not they have the characteristics similar to natural blastocysts. This study may be important to reveal the mechanisms of early germ and stem cell differentiation, and it holds great promises for advancing reproductive and developmental engineering technologies in the future.

2. MATERIALS AND METHODS

2.1. Animals and materials

Both male and female CD-1 mice were purchased from Charles River and maintained on a 16–8 h light-dark cycle. All procedures for animal use were approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University and every effort was made to minimize animal suffering. All Materials were purchased from Sigma unless specifically mentioned otherwise.

2.2. Preparation of mouse embryonic fibroblasts (MEFs)

On day 13.5, pregnant female mice were euthanized by cervical dislocation, and the intact uterus was extracted and cut into sections for each embryo. All organs from each embryo were totally removed using forceps and syringes. The embryo bodies were minced in the presence of trypsin. The minced tissue was transferred into a 15 ml conical tube with 5 ml of culture medium and centrifuged. The supernatant was aspirated and the tissue washed with 1× PBS. The pellet was re-suspended into 2 ml of MEF culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S). The mixture of the mouse embryonic fibroblast cells (MEFs) and medium was then transferred into tissue culture dishes with fresh culture medium and placed in humidified 5% CO₂ air at 37 °C. The MEFs were treated with mitomycin C (Chemicon) for 2 h and cultured in gelatin-coated dishes, for using as the feeder layer to culture embryonic stem cells.

2.3. Embryonic stem cell culture and derivation of blastocyst-like cell aggregates (BLCAs)

R1 murine embryonic stem cells (mESCs) either with or without green fluorescence protein (GFP) from ATCC were cultured in stem cell culture medium consisting of knockout DMEM supplemented with 15% knockout serum replacement (Invitrogen), 0.1mM β-mercaptoethanol (βME, Gibco), 2mM L-glutamine, antibiotics (1% penicillin/streptomycin), and 1000 U ml⁻¹ leukemia inhibition factor (LIF, Millipore). Feeder layer free culture of

ESCs were conducted using 0.1% gelatin-coated T-75 flask in humidified 5% CO₂ air at 37 °C¹¹.

As illustrated in Fig. 1a, to differentiate blastocyst-like cell aggregates (BLCAs) from the mESCs, single/detached mESCs (1×10⁶ cells/ml, obtained by treating the mESC aggregates cultured in T-flasks with 0.25% trypsin) were cultured in 10 ml of differentiation medium consisting of DMEM supplemented with 15% fetal bovine serum, 1% non-essential amino acid, 0.1mM 2-mercaptoethanol (βME), and antibiotics (1% P/S) in a 100 mm non adherent culture dish for 5 days. The number of BLCAs was then counted under microscope after differentiation for 5 days.

2.4. Characterization of BLCAs

For qRT-PCR analysis, RNAs were extracted from BLCAs and mESCs using an RNeasy plus mini kit (Qiagen) following the manufacturer's instructions. Next, reverse transcription was carried out to generate complementary DNA (cDNA) using the iScript™ cDNA synthesis kit (Bio-Rad) and Gene Amp 9700 PCR system. The qRT-PCR was conducted with the superfast SYBR Green mix (Bio-Rad) using a Bio-Rad CFX96 real time PCR system. Relative gene expression was determined using the CT method built in the Bio-Rad software. Genes for early germ cell and mESC pluripotency markers (Oct-4 and Sox2) and trophoblast (Cdx2) were studied with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) being used as the housekeeping gene. The primers used for all the genes are given in Table S2.

For immunohistochemical staining of marker proteins (Oct4, Sox2, SSEA-1 and Cdx2), mESCs (Oct4 and SSEA-1), BLCAs (Oct4, Sox2, and Cdx2), and murine embryonic stem-like cells (Oct4 and SSEA-1) derived from BLCAs were fixed in 4% paraformaldehyde and washed 3 times with 1x phosphate-buffered saline (PBS). For Oct4 and Sox2 staining, the samples were further permeabilized with 0.25% Triton X-100. The samples were then incubated in 3% bovine serum albumin (BSA) in 1x phosphate buffered saline with tween-20 (PBST) (1x PBS and 0.05% Tween 20) at room temperature for 1 hour to block non-specific binding, followed by an overnight incubation at 4 °C with primary antibodies (Abcam) targeting Oct-4, Sox2, Cdx2, or SSEA1. Afterward, the samples were washed 3 times and incubated in the dark at room temperature for 45 min with secondary antibodies (Abcam) including DyLight488 conjugated rabbit IgG (for Oct4, Sox2, and Cdx2) and DyLight 550 conjugated mouse IgM (for SSEA-1). All the antibodies were diluted in 3% BSA in 1x PBST according to the manufacturer's instruction. The samples were then washed and stained for nuclei using Hoechst 33342 (5 μM) for further microscopic examination.

2.5. BLCA transfer

BLCAs derived from mESCs either with or without GFP were transferred into the uterus of day-3.5 pseudo-pregnant CD1 female mice that were obtained through mating to vasectomized CD1 males. The pseudo-pregnant CD1 females were anaesthetized by isoflurane (2–5%) inhalation after a subcutaneous injection of buprenorphine (0.075 mg/kg body weight) before surgery. After 21 days of BLCA transfer using the standard methods

detailed elsewhere^{12,13}, uteri were collected, formalin fixed, and paraffin embedded for further histological analysis.

2.6. Statistical analysis

One-way ANOVA was conducted using the Statistical Analysis System (SAS) program to determine the *p* value between various treatments. The differences were taken as significant when the *p* value was less than 0.05.

3. RESULTS AND DISCUSSION

Although it has been recognized that successful blastocyst derivation from stem cells can greatly contribute to the development of reproductive medicine, an established method for the derivation is yet to be devised. To address this issue, we proposed a simple and efficient method for blastocyst derivation from murine embryonic stem cells. As illustrated in Fig. 1a, the single mESCs were observed to form embryoid bodies (EBs) in 3 days and further differentiate into BLCAs on day 5. Typical images of the single mESCs, EBs, and BLCAs on days 0, 3, and 5 are shown in Fig. 1b. The mESC-derived BLCAs were morphologically similar to natural blastocyst with inner cell mass-like cell aggregates enclosed in a trophoblast-like cell envelope¹⁴. The average number of BLCAs derived was 13 ± 2 per 1×10^6 single mESCs. The blastocyst consists of an inner cell mass (ICM), which subsequently forms the offspring, and a trophoblast (the outer layer of the blastocyst), which further develops into the placenta. Since the BLCAs differentiated from murine embryonic stem cells possess the same morphological configuration of a natural blastocyst, including an ICM and a trophoblast, we further characterized the BLCAs in terms of protein and gene expression. As shown in Fig. 2a, the BLCAs were positive for Oct4 (inner cell mass protein marker), Sox2 (inner cell mass protein marker), and Cdx2 (trophoblast protein marker) staining. Further quantitative studies of gene expression showed that expression of the trophoblast gene marker (*Cdx2*) was significantly (> 40 times) higher, while the expression of pluripotency gene markers (*Oct4* and *Sox2*) were significantly (more than 50 and 30 times for *Oct4* and *Sox2* genes, respectively) lower in BLCAs, than in mESCs (Fig. 2b). This is reasonable, since the BLCAs are differentiated from the mESCs. These results are also consistent with previous literature, which states that *Cdx2* is expressed in the trophoblast of blastocysts, and that *Oct4* and *Sox2* are expressed in the ICM.^{15,16,17}

When culturing the BLCAs on the feeder layer of inactivated mouse embryonic fibroblasts (MEFs) in a stem cell culture medium, inner cell mass-like cells (ICLCs) (arrow in Fig. 3a) could be obtained in 2 days. Moreover, embryonic stem-like cells (ESLCs) could be generated through a further culture of the ICLCs in a stem cell culture medium on the feeder layer of inactivated MEFs, with passaging every 5 days. The typical morphology of the ESLCs obtained at passages 1 and 2 is shown in Fig. 3a. The pluripotency of the ESLCs was confirmed by the positive staining of pluripotency protein markers, including Oct4 and SSEA-1 (Fig. 3b). These results suggest that the BLCAs derived from mESCs using our approach have characteristics similar to those of natural blastocysts from which embryonic stem cells could be derived through culturing and passaging.^{18,19,20}

We further examined the *in vivo* functionality of the BLCAs differentiated from mESCs by transferring the BLCAs into pseudo-pregnant mice. An average of 6.8 and 6.6 BLCAs (Table S1) were transferred into the right and left uterus, respectively, of 10 pseudo-pregnant female mice. Five of the mice were transplanted with BLCAs derived from mESCs with GFP, and the other five, with BLCAs derived from mESCs without GFP. As shown by the histological and fluorescence microscopy data (Fig. 4), the BLCAs successfully penetrated the endometrium and integrated with the native endometrium; however, no pups were born. These data suggest that the trophoblast of the BLCAs may function (i.e., penetrate the endometrium of uterus wall) properly; however, the ICM may not be the same as that in natural blastocysts for developing into the fetus.

4. Conclusions

In summary, we developed a convenient 3D suspension culture approach for deriving BLCAs from mESCs and showed that they had most of the characteristics of natural blastocysts, including the ability to penetrate into the uterine wall after *in vivo* transfer. However, further studies are needed to identify which specific factors and genes induce the fast differentiation of mESCs into BLCAs. In addition, further studies are also needed to optimize the culture system to provide a microenvironment similar to the *in vivo* niche of early embryo development for obtaining BLCAs capable of forming fetuses after being transferred into the uterus. Overall, our study may offer important insights into the development of early embryos and pluripotent stem cells. Moreover, it may provide a simple and fast approach to isolate trophoblast cells for studying the native microenvironment of pluripotent stem cells without the need of sacrificing animals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Derivation of blastocyst-like cell aggregates (BLCAs) from murine embryonic stem cells (mESCs) by suspension culture. (A) A schematic illustration of the procedure for deriving BLCAs from mESCs. The mESCs were cultured in stem cell medium in T-75 flask for 4 days and then dissociated into single cells with light trypsinization. The single cells (1×10^6) were cultured in differentiation medium in 100 mm nonadherent culture dish to form embryoid bodies (EBs) within 3 days and further differentiated into BLCAs consisting of inner cell mass-like cells in the core and trophoblast-like cells in the shell on day 5. (B) Typical micrographs showing the morphology of single mESCs (arrow) suspended in a 100 mm nonadherent culture dish on day 0 (I), EBs formed on day 3 (II), and BLCAs on day 5 (III). Scale bar: 50 μ m.

Fig. 2. Characterization of BLCAs derived from mESCs in terms of protein and gene expression. (a) Immunostaining of BLCAs derived from mESCs with Oct-4 (ICM protein marker), Sox2 (ICM protein marker), and Cdx2 (trophoblast protein marker) showing their positive expression in the derived BLCAs. (b) Quantitative data on the expression of *Oct-4*, *Sox2*, and *Cdx2* gene markers in BLCAs versus control (non-differentiated) mESCs. Scale bar: 150 μ m. *: $p < 0.05$.

Fig. 3. Derivation and characterization of embryonic stem-like cells (ESLCs) from BLCAs differentiated from mESCs. (a) Colony-forming inner cell mass (ICM)-like cells (ICLCs) (arrow) were obtained after seeding BLCAs on the feeder layer of inactivated MEFs for 2 days and ESLCs created after passaging the ICLCs on the same feeder layer in stem cell culture medium and culturing for 5 days (passage 1) and 10 days (passage 2). (b) Immunostaining of pluripotency protein markers including Oct-4 and stage specific embryonic antigen (SSEA)-1 with in mESCs and the ESLCs derived from BLCAs. Scale bars: 150 μ m in (a) and 100 μ m in (b).

Fig. 4. Phase (a) and green fluorescence microscopy (b) images showing histology (hematoxylin and eosin) of uterine tissue at 21 days after transferring BLCAs derived from mESCs with GFP into the uterus of pseudo-pregnant mice. The BLCAs penetrated into the uterine tissue and integrated with the native tissue *in vivo*. Scale bar: 100 μ m.